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Carcinoma: Focusing MHC/Peptide Complexes to Lipid Rafts

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Genetic engineering of tumor cells to express MHC class II and subsequent use of said cells for treatment of established and metastatic tumors has yielded promising results in animal models for treatment of breast cancer. It is widely believed that the vaccine efficacy is due to the ability of such tumor cells to present tumor-specific antigens to CD4⁺ T helper cells which activate the immune system to eradicate tumors. Next generation cell-based vaccines will have enhanced antigen presentation capabilities to further stimulate the anti-tumor immune response. It has recently been proposed that MHC class II molecules physically localize to cell-surface microdomains, termed lipid rafts, to enhance antigen presentation. Further more, a correlation has been observed where cell-based tumor vaccines that have high levels of MHC class II in such rafts have higher efficacy than those with diminished or abolished levels of MHC class II in rafts. We propose to further target MHC class II molecules to lipid rafts to enhance the antigen presentation capabilities of tumor cell-based vaccines and than to use these modified vaccine cells for the treatment of established, metastatic disease in mouse models of breast cancer.

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Introduction

Genetic engineering of tumor cells to express MHC class II and subsequent use of said cell-based vaccines for treatment of established and metastatic tumors has yielded promising results in animal models for treatment of breast cancer. It is widely believed that the vaccine efficacy is due to the ability of such tumor cells to present tumor-specific antigens to CD4⁺ T helper cells which activate the immune system to eradicate tumors. Next generation cell-based vaccines will have enhanced antigen presentation capabilities to further stimulate the anti-tumor immune response. It has recently been proposed that MHC class II molecules physically localize to cell-surface microdomains, termed lipid rafts, to enhance antigen presentation. Further more, a correlation has been observed where cell-based tumor vaccines that have high levels of MHC class II in such rafts have higher efficacy than those with diminished or abolished levels of MHC class II in rafts. We propose to further target MHC class II molecules to lipid rafts to enhance the antigen presentation capabilities of tumor cell-based vaccines and then to use these modified vaccine cells for the treatment of established, metastatic disease in mouse models of breast cancer.

Body

Note: Text appearing in the original statement of work (SOW) is underlined

Task 1: Generation and characterization of MHC class II and cysteine substituted MHC class II positive mammary carcinoma cells, Months 1-12

- a. Create point mutations in the DNA encoding the cytoplasmic domain of the beta chain of I-A^d that substitute cysteine residues for wild type amino acids and subclone, along with the alpha chain of I-A^d into a bicistronic expression plasmid (pIRES). Create similar expression vectors using wild type beta chain DNA. **Previously Reported**
- b. Transfect 4T1/CD80 mammary carcinoma cells with MHC class II expression plasmid from task 1a. Limit dilution clone to establish 3-5 clones of each transfectant. **Previously Reported**
- c. Characterize expression of I-A^d on each cell line using flow cytometry. Check for positive expression of CD80 and endogenous MHC class I molecules. **Previously Reported**
- d. Assess MHC class II activity on transfected 4T1 cells by assessing stable dimer formation by western analysis. Test the ability of transfectants to present synthetic peptides to peptide-specific I-A^d-restricted T cells *in vitro*.

Results: Formation of stable MHC class II heterodimers is essential for functional antigen presentation. MHC class II heterodimers are resistant to SDS-dissociation and can be detected using western analysis. Unfortunately, the most reliable antibody for detecting I-A^d, called KL295, did not detect stable dimers of I-A^d in any 4T1 cell line or control lysates of BALB/c splenocytes and therefore western analysis can not be performed to determine stability of mutant forms of I-A^d. Another method for determining stable dimer formation is the ability of cells expressing MHC class II to present synthetic peptides to antigen-specific, I-A^d-restricted T cell hybridomas *in vitro*. 4T1/A^d wt, 4T1/A^d2cys, and 4T1/A^dLAT cells were pulsed with synthetic OVA peptide 323-339 and the corresponding T cell hybridoma DO11.10. As is shown in figure

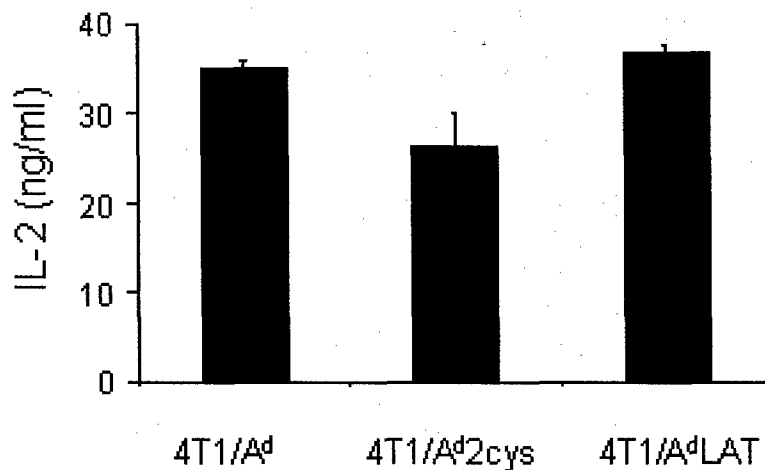


Figure 1. Cysteine-substituted I-A^d molecules form functional heterodimers. 5×10^4 4T1 cells were mixed with 50 ng/ml OVA peptide 323-338 and 5×10^4 DO11.10 hybridoma cells and cultured overnight. IL-2 release was quantified by ELISA.

1, all cells successfully presented peptide to the hybridoma, as measured by IL-2 release from activated hybridoma cells. Therefore, cysteine mutations introduced into the cytoplasmic domains of I-A^d molecules do not impact stable dimer formation.

Final Result for Task 1 – Completed

Task 2: Characterization of MHC class II-lipid raft interactions in transfected mammary carcinoma cells, Months 12-18

- a. Treat the wild type and cysteine substituted MHC class II positive 4T1 cells established in task 1 with a variety of detergents at different concentrations to isolate detergent insoluble lipid rafts. Quantify MHC class II content by trace densitometry following western blot analysis of both lipid raft and plasma membrane fractions of detergent treated cells.

Previously we have reported that cysteine substituted I-A^d molecules expressed by 4T1 cells enhanced localization of I-A^d to lipid rafts. As enhanced localization to rafts is absolutely necessary if tumor cell-based vaccine efficacy is to be increased, we rigorously tested if the mutant I-A^d localization to rafts was enhanced by varying both levels of detergent and total number of cells in detergent extraction procedures. As is shown in figure 2A, varying the levels of detergent and cell number alone greatly affected the levels of I-A^d detected in the lipid raft fractions from 4T1 cells expressing wild type I-A^d. Because such small differences in detergent extraction conditions can greatly alter our results, many experiments were conducted with all 3 4T1 cell lines to determine raft localization of mutant MHC class II. Over the course of several experiments in which cell numbers and detergent concentrations remained constant, we do not detect a noticeable difference in either raft localization of the total pool or cell-surface localized mutant I-A^d molecules (figure 2B). Therefore, we unfortunately have to conclude that cysteine

substituted MHC class II molecules lack the ability to enhance localization of MHC class II to lipid rafts.

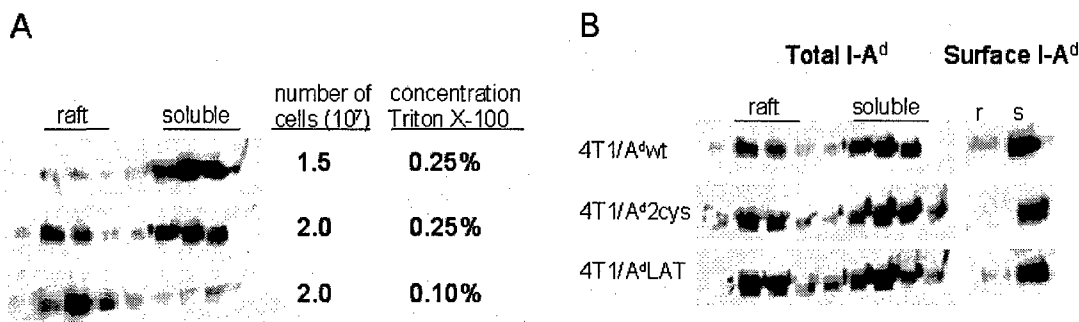


Figure 2. *A.* Small changes in detergent extraction conditions alter raft localization of wild type I-A^d. The indicated number of 4T1/A^dwt cells were lysed in the indicated amount of Triton X-100 and lysates were fractionated on a discontinuous sucrose gradient. Gradient fractions were analyzed by western analysis for I-A^d. Lipid raft fractions and soluble membrane fractions are indicated. *B.* Cysteine substituted I-A^d does not enhance raft localization. 2.0×10^7 cells were lysed in 0.25% Triton X-100 and analyzed as in (A). Prior to lysis, cell surface proteins were labeled with biotin. Raft fractions and soluble membrane fractions were precipitated with streptavidin-sepharose to isolate cell-surface proteins and analyzed for I-A^d by western analysis.

- b. Determine palmitate incorporation into wild type and mutant MHC class II molecules by incubating cells with radio-labeled palmitate, followed by immunoprecipitation of MHC class II and measurement of incorporated palmitate.

Results: Cysteine residues in amino acids can be palmitoylated to enhance raft localization. To determine if palmitate had incorporated into cysteine substituted I-A^d molecules, we had proposed to incubate cells with radio-labeled palmitate and immunoprecipitate I-A^d to check for incorporated palmitate. This experiment proved to be technically difficult owing to the long exposure time for the detection of tritiated palmitate. Another acceptable method for measuring palmitate incorporation into raft localized proteins is to use an inhibitor of palmitoylation, 2-bromopalmitate, which has been shown to block raft localization. As is shown in figure 3, 2-bromopalmitate had no effect on raft localization of I-A^d in the 4T1/A^d/2cys cell line indicating that cysteine substitution did not occur. This data combined with the data from task 2A give a strong indication that cysteine mutations introduced into the cytoplasmic domains of MHC class II do not enhance lipid raft localization.

Final Result for Task 2 - completed

Task 3: Examination of immunogenicity of non-mutated and cysteine-substituted MHC class II+ 4T1 mammary tumor cell vaccines, Months 12-30.

Has not been attempted yet

Task 4: Quantification of antigen presentation capacity of MHC class II+ and cysteine substituted MHC class II+ mammary carcinoma tumor cell vaccines, Months 18-36.

- a. 4T1 vaccine cells from task 1 will be transfected with a plasmid containing the cDNA for OVA under the control of a β -actin promoter. OVA expression will be confirmed using flow cytometry. Stable OVA+ cell lines will be established by limiting dilution cloning.
- b. Test in vitro antigen presentation using T cells from OVA TCR transgenic mice and 4T1 targets from task 4a. TCR transgenic T cells will be adoptively transferred into a non-transgenic mice and either vaccinated with OVA protein in adjuvant or vaccinated with adjuvant alone. Three weeks following vaccination, CD4 T cells will be isolated and tested for the ability to respond to MHC class II positive vaccine cells in vitro. Cytokine release (IL-2, IL-4, and IFN γ) will be quantified by ELISA.

During the onset of Task 4, other members of the laboratory determined that 4T1 cells expressing I-A^d and endogenously synthesized ovalbumin did not process the epitope recognized by the DO11.10 T cell receptor (Clements and Ostrand-Rosenberg unpublished data). Therefore, task 4 can not be completed as proposed due to technical difficulties.

As an alternative experiment, we asked if cysteine substituted I-A^d expressing 4T1 cells could present OVA peptide 323-339 to DO11.10 transgenic T cells. Transgenic T cell responses are more relevant than responses generated by the corresponding T hybridoma cells in Task 1d as hybridomas have been selected for antigen responses and have been previously primed to antigen, whereas T cells from the DO11.10 TCR transgenic mouse are naïve and their activation is more indicative of true *in vivo* T cell responses. As is shown in figure 3, peptide presentation to DO11.10 T cells is not enhanced by expression of cysteine substituted I-A^d in 4T1 cells.

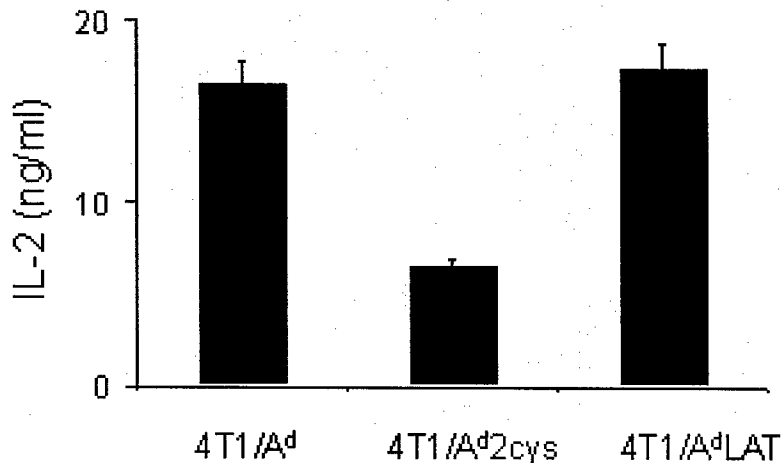


Figure 4. Cysteine substituted I-A^d does not enhance antigen presentation to T cells. 5×10^4 4T1 cells were mixed with 50 ng/ml OVA peptide 323-338 and 5×10^4 DO11.10 hybridoma cells and cultured overnight. IL-2 release was quantified by ELISA. All 4T1 cells also expressed CD80.

Final Report for Task 4 – completed

Additional Work

While pursuing the above experiments, I preformed additional experiments with a second murine tumor cell-based vaccine system designed to address the role of dendritic cells (DC) in the response to vaccine cells. We have long proposed that tumor cells directly activate CD4 T cells, however, we found that conditional deletion of dendritic cells in mice restored tumorigenicity of sarcoma cells expressing the MHC class II molecule I-A^k (Sal/A^k) cells, indicating that DC are necessary for vaccine efficacy (figure 5A). Concurrent *in vitro* experiments demonstrated that MHC II molecules could physically transfer from necrotic tumor cells to recipient DC (figure 5B). Furthermore, we show that MHC class II-antigen complexes, generated in the tumor cells, can transfer to DC and the DC can subsequently activate both T hybridoma cells and naïve TCR transgenic T cells (figure 5C). These experiments suggest that vaccine efficacy provided by MHC class II⁺ tumor cells is the result of transfer of the immunogenic antigen-MHC class II complex to DC which in turn activate T cells.

We wish to extend these experiments to the 4T1 mammary carcinoma system and determine if both MHC class I and II molecules can transfer from dead 4T1 cells to DC. If so, we hypothesize that DC recipient of 4T1 MHC molecules could be used as an immunotherapy for established metastatic disease. These data could provide an additional rationale for DC-based immunotherapies and eliminate the need to use live tumor cells as therapeutic agents.

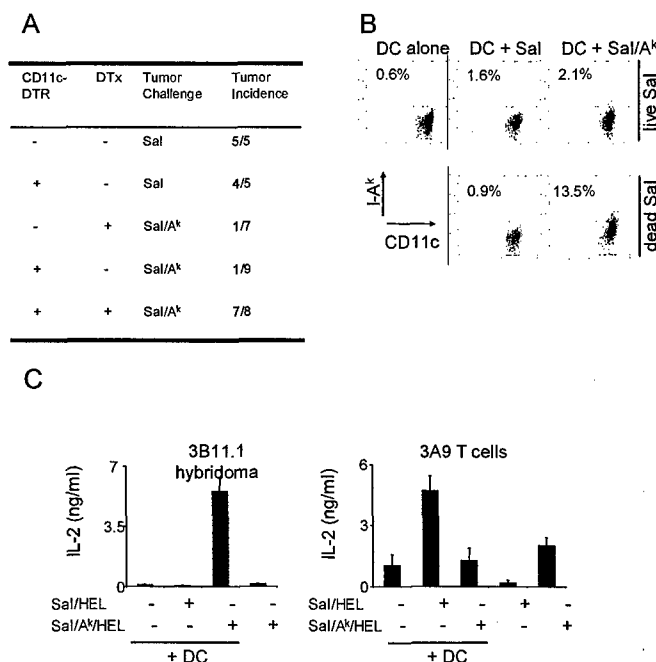


Figure 5. MHC class II-antigen complexes transfer from tumor cell-based vaccines to DC. **A.** Transgenic mice whose DC express the simian diphtheria toxin receptor were challenged with diphtheria toxin or left untreated before inoculation with Sal or Sal/A^k cells and tumor incidence recorded. **B.** Live or dead Sal or Sal/A^k cells were mixed with splenic DC isolated from FVB (H-2^q). DC were then analyzed by flow cytometry for the DC marker CD11c and I-A^k. **C.** Sal/HEL and Sal/A^k/HEL cells were lysed and mixed with splenic FVB DC. DC were then incubated with A^k-HEL specific 3B11.1 hybridoma cells or 3A9 transgenic T cells. IL-2 release the following day was determined by ELISA.

Key research accomplishments

- 4T1 cells engineered to express wild type and cysteine substituted I-A^d molecules have been assessed for MHC class II localization to lipid rafts: no enhanced localization of MHC class II to lipid rafts was observed using standard biochemical techniques or cell-based assays to measure antigen presentation.
- Using a second cell-based vaccine system, we have demonstrated that MHC class II molecules can physically transfer from tumor cells to DC and such transfer may explain the necessity of DC for effective vaccination. Currently, we are extending these studies using 4T1 cells.

Reportable outcomes

A short talk entitled "Dendritic cells acquire functional peptide-MHC complexes from necrotic tumor cells" was given at the Keystone Symposia Meeting on Inflammation and Cancer in February of 2005 in Breckenridge CO.

Conclusions

The purpose of this project was an attempt to improve vaccine efficacy of MHC class II⁺ tumor cell-based vaccines and apply such improvements to the treatment of mammary carcinoma in animal models. We proposed that targeting MHC class II molecules to lipid rafts would enhance cell-based vaccine antigen presentation and yield more potent immunotherapies. However, lipid raft localization mutations introduced into MHC class II molecules did not enhance localization of MHC class II to lipid rafts. We have therefore elected to forgo vaccination studies with such cell-based vaccines as they do not have enhanced antigen presentation capabilities over existing therapies.

Additional experiments have demonstrated that DC are necessary for effective vaccination with tumor-cell based vaccines and we propose that MHC-antigen complexes transfer from tumor cells to DC for T cell stimulation. This finding is not only important for future vaccine development, but suggests a novel mechanism for *in vivo* antigen presentation. We propose to exploit this function of DC for the creation of new therapies for the treatment of breast cancer.